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Structural characterization of the P_{CO/O_2} compound of cytochrome *c* oxidase

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Abstract **The structural properties of a key transient oxygen intermediate of cytochrome *c* oxidase, P_R, remain an enigma, although inferences have been drawn from its equilibrium analogues, P_{CO/O_2}, P_H and P_M. With resonance Raman spectroscopy, an oxygen isotope-sensitive band at 806 cm^{-1} was observed in P_{CO/O_2} produced by adding CO and O_2 to the resting enzyme. The vibrational band shifted to 771 cm^{-1} upon isotopic substitution of $^{16}O_2$ with $^{18}O_2$. The same modes at 806 and 771 cm^{-1} were present simultaneously when the mixed isotope, $^{18}O^{16}O$, was employed, indicating that in P_{CO/O_2} the O–O bond is cleaved, resulting in a $Fe^{4+}{=}O^{2-}$ structure. This result unifies the nature of the three equilibrium analogues of the P_R intermediate.**

Keywords: Raman; Biophysics; Proton pumping; Ferryl; Bioenergetics

1. Introduction

The reaction of cytochrome *c* oxidase (C*c*O) involves an oxidative phase, in which the four-electron reduced enzyme is fully oxidized by molecular oxygen, and a reductive phase, in which the enzyme is re-reduced by cytochrome *c* [1,2]. The release of energy from the reduction reaction of oxygen to water during the oxidative phase is harnessed by the membrane-bound enzyme for proton translocation against the pH gradient. C*c*O consists of four metal redox centers: Cu_B and heme a_3, making up the binuclear center (where the reduction of oxygen occurs), and Cu_A and heme *a*, mediating electron transfer from cytochrome *c* to the binuclear center.

Several transient intermediates of C*c*O, including A, P_R, F and H, have been identified during the reduction reaction of oxygen to water. Apart from P_R, the properties of all the oxygen intermediates are moderately well characterized. Intermediate A (Fe^{2+}–O_2) is an oxy species formed by ligation of O_2 to the ferrous heme a_3. Intermediate F ($Fe^{4+}{=}O^{2-}$) is a ferryl compound, in which the O–O bond has been cleaved. Intermediate O_H (Fe^{3+}–OH) is a hydroxide intermediate formed in the last step of the oxidative phase [3]. In contrast, the structural properties of P_R, originally believed to be a peroxo species with an intact O–O bond, are not well established due to its rapid decay at neutral pH and room temperature. As the $P_R \rightarrow F$ conversion is the first proton pumping step in the oxidative phase [4], understanding the structure properties of the P_R intermediate is indispensable for evaluating how proton pumping is gated by this important enzyme.

The difference spectrum of P_R with respect to the fully oxidized enzyme exhibits a characteristic absorbance maximum at 607 nm [5]. Three analogs of the P_R intermediate with the same optical properties have been made on the bench: (1) P_{CO/O_2}, which is made by purging the oxidized C*c*O with a mixture of CO and O_2 gas at high pH, (2) P_H, which results from the reaction of oxidized C*c*O with H_2O_2 at high pH, and (3) P_M, which is made by mixing the mixed-valence (two-electron reduced) form of C*c*O with O_2 [6–8]. Upon careful examination of P_H, the original assignment that P_R as a peroxo intermediate was challenged by Weng and Baker [8], who proposed that it was a ferryl species with a broken O–O bond as is F rather than a peroxo species. They proposed that a radical center, presumably located in the Cu_B site, was present in P_H, due to electron transfer to the heme a_3 center required for the O–O bond cleavage. Although both P and F intermediates are both ferryl species, they postulated that the presence of the radial center in P accounted for its distinct optical transitions at 607 nm, with respect to the 580 nm transition in F [8]. The firm assignment of compound P_H as a ferryl species was made later by Kitagawa and co-workers [9] with resonance Raman spectroscopy. By tuning the excitation laser to 607 nm, they selectively enhanced all the modes associated with the compound P_H produced by mixing the resting C*c*O with H_2O_2. An oxygen isotope-sensitive mode was identified at 769 and 803 cm^{-1} for the $H_2{}^{18}O_2$ and $H_2{}^{16}O_2$ derivatives, respectively. When $H_2{}^{16}O^{18}O$ was used, both bands at 769 and 803 cm^{-1} were observed simultaneously with half of the intensities of the bands seen when either $H_2{}^{18}O_2$ or $H_2{}^{16}O_2$ was used. This indicates that P_H cannot be a peroxo compound, otherwise a single peak located in between 769 and 803 cm^{-1} would be present. More recently, Palmer, Gennis, and colleagues [10] demonstrated that P_M generated by mixing mixed-valence C*c*O with $^{18}O_2$ in $H_2{}^{16}O$ solution is also a ferryl species by examining ^{18}O enrichment in the reaction mixture with mass spectrometry.

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Abbreviations: EPR, electron paramagnetic resonance; C*c*O, cytochrome *c* oxidase; P_R, P_M, P_H, P_{CO/O_2}, the P species in cytochrome *c* oxidase formed in the reaction of the fully reduced enzyme with oxygen, formed in the reaction of the mixed-valence enzyme with oxygen, formed by the reaction of hydrogen peroxide with the fully oxidized enzyme and formed by the reaction of CO and O_2 with the fully oxidized enzyme

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In contrast to P_H and P_M, there is no evidence proving that P_{CO/O_2} is a ferryl species, although it has similar spectroscopic features including a similar optical transition and a similar ^{16}O–^{18}O isotope shift as P_H [11]. Here, we aimed to investigate the number of the oxygen atoms bound to heme a_3 in P_{CO/O_2} by mixing the resting C*c*O with a mixture of CO and various isotopes of O_2 at pH 8.5.

2. Materials and methods

C*c*O samples were purified from beef hearts by the method described by Yoshikawa [12]. All the measurements were performed with freshly purified C*c*O without previously being frozen. The enzyme concentration was determined by the optical absorption difference between the fully reduced enzyme at 604 nm minus that of fully oxidized enzyme at 630 nm with 23.3 mM^{-1} cm^{-1} as the extinction coefficient difference. Oxygen isotope gases ($^{18}O_2$ and $^{18}O^{16}O$) were purchased from ICON (Summit, NJ). The $^{18}O_2$ purity is >99% and the composition of $^{18}O^{16}O$ bottle was determined by Raman spectroscopy to be 40% $^{18}O_2$, 40% $^{18}O^{16}O$, and 20% $^{16}O_2$ based on the relative intensities of the ν_{O-O} modes at 1465, 1511 and 1554 cm^{-1}, respectively.

To make P_{CO/O_2}, the resting enzyme was first diluted with the 0.2 M Tris–Cl buffer (pH 8.5) in the presence of 0.1% *n*-decyl-β-maltoside in a Raman cell. The Raman cell was then sealed with a septum and purged with Argon for ~20 min to remove the air. Subsequently, 500 μl of $^{16}O_2$, $^{16}O^{18}O$ or $^{18}O_2$ was injected along with 500 μl of CO into the sealed cell to form the P_{CO/O_2}. The final concentrations of C*c*O and each gas were ~50 and ~500 μM, respectively. The formation of P_{CO/O_2} in each sample was confirmed by the appearance of 607 nm band in the absorption difference spectrum with respect to the fully oxidized C*c*O.

The Raman measurements were performed with previously described instrumentation [13]. The excitation source was a He–Cd laser with an output of 441.6 nm. The incident light power on the spinning sample was 1 mW and the acquisition time for each spectrum was ~2 h. After each Raman measurement, the integrity of the P_{CO/O_2} sample was confirmed by optical absorption spectroscopy.

3. Results

Fig. 1 shows the optical absorption spectra of P_{CO/O_2} and fully oxidized bovine C*c*O. The difference between them, depicted in the inset, exhibits the characteristic Compound P absorbance bands at 414, 439 and 607 nm. This P species was stable for more than 12 h. The static Raman spectra of the P_{CO/O_2} derivatives for $^{18}O_2$, $^{18}O^{16}O$, and $^{16}O_2$ are shown

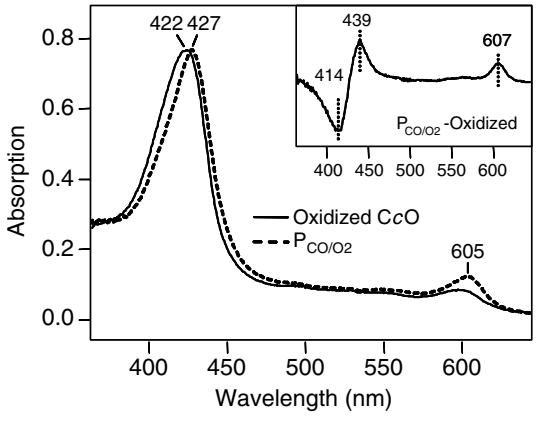

Fig. 1. The optical absorption spectra of P_{CO/O_2} (dashed line) formed by mixing oxidized C*c*O with CO and O_2. It is compared with that of the fully oxidized enzyme (solid line). Their difference spectrum is shown in the inset.

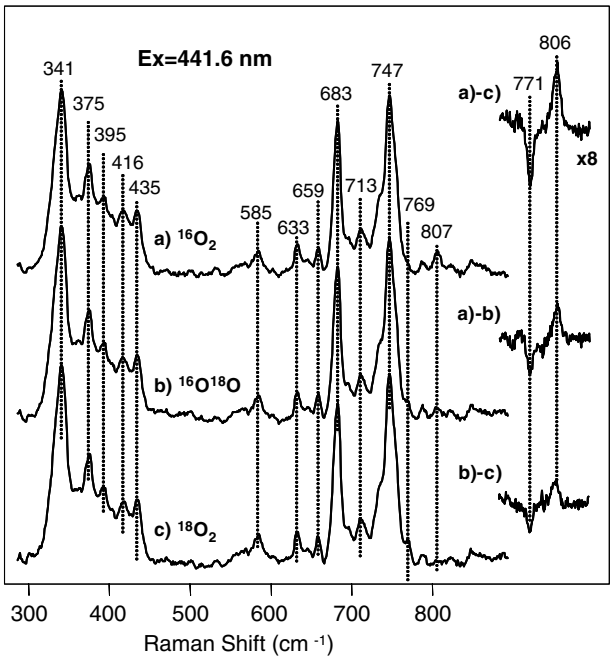

Fig. 2. The resonance Raman spectra of P_{CO/O_2} formed by mixing oxidized CcO with CO and O_2. The O_2 isotopes used were $^{16}O_2$ (a), $^{16}O^{18}O$ (b) and $^{18}O2$ (c). The subtractions between these three spectra were performed and shown as labeled.

in Fig. 2 as traces a, b and c, respectively. The only observable spectral difference as the sample treatment changes from $^{16}O_2$ to $^{18}O^{16}O$, and to $^{18}O_2$ is a systematic intensity increase at 769 cm^{-1} that is concurrent with the loss of intensity at 807 cm^{-1}. This spectral change is more clearly demonstrated in the normalized difference spectra shown on the right in Fig. 2. In the top trace, it is evident that the oxygen isotope-sensitive band is located at 806 cm^{-1}, which shifts to 771 cm^{-1} upon the substitution of $^{16}O_2$ with $^{18}O_2$. Most importantly, the bottom two difference spectra show that when $^{18}O^{16}O$ was used, no additional band located in between 771 and 806 cm^{-1} was detected. If P_{CO/O_2} is a peroxo species, a negative and positive band located in between 771 and 806 cm^{-1} would be expected in trace b and c in Fig. 2, respectively. Since no band at intermediate frequency was observed, we conclude that P_{CO/O_2} is also a ferryl species with only a single oxygen atom bound to the heme a_3, the same as P_H and P_M. Further intensity analysis of the normalized spectra of the reaction products shows that the a–b difference spectrum is 60% of that in a–c difference spectrum and the b–c difference spectrum is 40% of the a–c difference spectrum, consistent with the expectations based on the composition of the mixed labeled O_2 gas (40% $^{18}O^{18}O$, 40% $^{18}O^{16}O$ and 20%$^{16}O^{16}O$). It corroborates that P_{CO/O_2} possesses only one oxygen atom. Moreover, the Fe=O stretching frequencies detected here for P_{CO/O_2} are similar to those of P_H reported by Kitagawa's group [9], indicating that all the P_R analogs, including P_M, P_H and P_{CO/O_2}, are the same ferryl species.

4. Discussion

The resonance Raman data presented here unifies some of the structural properties of the three equilibrium P analogues,

Table 1
Properties of the putative ferryl species in the P_R and F intermediates and in the equilibrium analogues of P_R in C*c*O

Compound/intermediate	Optical transition (nm)	Raman mode frequency (cm^{-1})	EPR signal
P_R	607	804 (a)	Cu_B^{2+}—Fe^{4+}=O^{2-} (f)
P_M	607	804 (b)	ND
P_H	607	804 (c)	Tyr-129 (g)
P_{CO/O_2}	607	806 (d)	ND
F	580	786 (e)	ND

References: (a) [22]; (b) [7]; (c) [9]; (d) this work; (e) [23]; (f) [17]; (g) [21].

P_{CO/O_2}, P_H and P_M (see Table 1), and at the same time it underscores the essential need to do direct oxygen isotope measurements on the P_R intermediate that is formed during the catalytic cycle. The hypothesis that P_R is a ferryl instead of a peroxo species has been inferred from the assumption that P_R has the same structural features as its equilibrium analogs. However, considering the fact that distinct conditions are applied for producing these equilibrium P analogs, the analogous optical transition is not enough to validate the assumption that they are the same species as P_R. Indeed, Einarsdottir et al. [14] reported that P_H, P_M and P_{CO/O_2} are all the same but are different from P_R on the basis of spectral deconvolution of the time-dependent data obtained during the oxygen reaction of the fully reduced enzyme.

The P_R species produced during the reaction of the fully reduced enzyme with oxygen exhibits an unusual electron paramagnetic resonance (EPR) signal that was attributed to Cu_B^{2+}, which was postulated to be weakly antiferromagnetically coupled to the Fe^{4+}=O^{2-} center ($S = 1$) [15–17]. This observation is consistent with the fact that during the oxygen chemistry of the fully reduced enzyme, a sufficient number of electrons are available from the redox centers and hence an additional electron from a nearby amino acid is not necessary, in contrast to the case of the equilibrium P-analogues, which are in a two-electron reduced state. In the conversion from P_R to F the EPR signal disappears presumably from the change in the ligand on Cu_B^{2+} due to the presence of an additional proton in the binuclear center. Recently, Wiertz et al. [18] reported the presence of two different types of radical species in P intermediates in freeze quenched C*c*O samples obtained during the reaction of the fully reduced enzyme with oxygen. They proposed that three different ferryl species, P_M, P_R and F, were formed sequentially ($P_M \rightarrow P_R \rightarrow F$) during the reaction [18]. It is important to note that in the equilibrium P-analogues, such as P_M, the enzyme is electron-deficient and hence the formation of an amino acid radical in the proximity of the binuclear center has been postulated to account for the electron needed for the formation of the ferryl moiety. The hypothesis put forth by Wiertz et al. [18] thus implies that the electron required for the O–O bond cleavage reaction is mediated by an amino acid residue in the vicinity of the binuclear center.

So far no radical species has been detected in P_M or P_{CO/O_2} with EPR spectroscopy. The silent EPR signal has been attributed to the hypothesis that an amino acid radical, likely on Tyr-244 (which is covalently linked to a Cu_B ligand via a posttranslational modification) is spin coupled to the Cu^{2+} center and the oxoferryl moiety itself is in a low spin ($S = 1$) state [7,19]. On the other hand, EPR radical signals have been detected in P_H in both the bovine and the *P. denitrificans* enzymes [17,20]. The EPR signal was attributed to the presence of radicals more distant from the Cu^{2+} center than Tyr-244, thereby eliminating the spin–spin coupling. In the former enzyme the radical species was originally attributed to a Trp residue, whereas that in the latter was assigned to a Tyr residue. Upon re-analyzing these EPR data, Svistunenko et al. [21] later proposed that the radical centers could be accounted for by Tyr-129 in the bovine C*c*O and the equivalent Tyr residue (Tyr-167) in the *P. denitrificans* enzyme. Although this proposal is mechanistically possible, if the radical center in P_H is in fact located remotely (~10 Å) from the binuclear center as proposed (presumably due to radical migration from Tyr-244), a conversion from the 607 nm species to the 580 nm species would be expected, since the difference in the optical properties of P (607 nm) and F (580 nm) has been postulated to result from the presence of a radical center in the proximity of the binuclear center in Compound P, but not in F [8]. In addition, the loss of the spin–spin coupling due to radical migration should have allowed the recovery of the unusual EPR spectrum of the $[Cu_B^{2+}]^{\cdots}[Fe^{4+}{=}O^{2-}]$ moiety as that observed in P_R. In the present study, we show that the 607 nm P_{CO/O_2} species exhibits a long lifetime (>12 h), yet the silence of the EPR spectrum suggests that the radical remains on Tyr-244. It is unclear why the radical center should migrate in P_H, but not in P_{CO/O_2} and P_M. It is also unknown why P_R exhibits a 607 nm optical transition despite the absence of a radical center near the binuclear center. Clearly, more experiments remain to be performed to reconcile these very diverse spectroscopic observations.

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